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Introduction

Breast cancer is the most common carcinoma of women in the United States, accounting for more than 40,000 American women's deaths annually. Targeted therapies, such as tamoxifen in estrogen receptor-positive breast cancer, have led to major important improvements in the outcomes of this cancer, but effective chemotherapy targets are lacking and the mortality rate of advanced breast cancer is still high. Aldo-keto reductase 1 B10 (AKR1B10) is overexpressed in tested human breast cancer tissues and mediates acetyl-CoA carboxylase-α (ACCA) stability, affecting fatty acid *de novo* synthesis and cell growth. This study is aimed to identify and evaluate AKR1B10 as a now target for the treatment of breast cancer. In this study, we will determine AKR1B10 expression in breast cancer, define the role of AKR1B10 in lipid metabolism, proliferation, and tumorigenicity of breast cancer cells using AKR1B10-targeted breast cancer cells and animal tumor models, and elucidate the regulatory mechanisms of AKR1B10 on lipid metabolism of breast cancer cells via identifying the functional domain(s).

Body

1) AKR1B10 silencing inhibits breast cancer cells BT-20 growth in culture and tumorigenesis in female nude mice.

Two siRNAs targeted to encoding (siRNA 1) and 3' untranslational (siRNA 2) regions of *AKR1B10* were chemically synthesized (Ambion, TX). A scrambled siRNA was used as a negative control. For siRNA delivery, BT-20 cells ($3.5 \times 10^{4\text{-}5}$ in Opti-MEM I medium) were mixed gently with siRNA and OligofectAMINE (Invitrogen, CA) in a volume of 0.5 ~ 1.5 ml and incubated at 37^{o}C, 5% CO_2 for 4 hours, followed by the addition of an equal volume of fresh medium containing 20% FBS. The siRNA and oligofectAMINE mixtures were prepared following manufacturer's instructions. As a result, *AKR1B10* silencing resulted in ~ 50% decrease of lipid synthesis, particularly in phospholipids critical for biomembrane synthesis, and remarkable inhibition of cell growth ($p<0.001$). The inhibition of lipid synthesis and cell growth induced by *AKR1B10* silencing in turn noticeably suppressed the tumorigenesis of BT-20 cells in female nude mice ($p=0.0462$). The BT-20 cells with *AKR1B10* silencing did not form any tumors in 6 female nude mice until 42 days while the scrambled siRNA control cells inoculated in the same mice all produced tumors within 25 days (a ratio of 6/0 of scrambled siRNA cells/AKR1B10 siRNA cells). Please see the enclosed manuscript for more details.

2) AKR1B10 is overexpressed in breast cancer and may promote tumor growth and progression.

To understand AKR1B10 expression in breast cancer and effects on tumor growth and progression and patient survival, a tissue microarray YTMA-23 that contains 246 primary breast cancer tissues from patients diagnosed from 1962 to 1983, with complete clinicopathological records and a mean follow-up time of 20 years was used in this study. Normal breast tissues were included as an internal control. Our results demonstrated that AKR1B10 was undetectable or at a very low level in normal breast lobules and ducts, but overexpressed in 184 (83.6%) of 220 infiltrating carcinomas, and more interestingly, AKR1B10 expression in malignant tissues positively correlated with tumor size ($p=0.0012$) and lymph node metastasis ($p=0.0123$), but reversely correlated with disease-related patient survival ($p=0.0120$), particularly in breast cancer at early stages (tumor size < 2 cm^3 without metastasis) ($p=0.0270$). Univariate ($p=0.0077$) and multivariate ($p=0.0192$) analyses indicated that AKR1B10 was a significant prognostic factor, alone or in combination with tumor size and lymph node status, for breast cancer. AKR1B10 expression in ductal carcinoma in situ (DCIS), recurrent tumors, and metastatic lymph nodes were also investigated and please see the enclosed manuscript for more details.

3) Fresh tumor, normal breast, and serum from the same subject are being collected to validate the potential of AKR1B10 as a serum marker.

Frequent upregulation and promoting role of AKR1B10 in breast cancer values it as a specific marker of breast cancer for targeting therapy, diagnosis, prognostic prediction, or therapeutic response estimates. Therefore, it will be invaluable if ARK1B10 is validated as a serum marker. To carry on this study, the matching tumor, normal adjacent breast, and serum collected before surgery are being collected and 50 pairs have been obtained and under investigation. Preliminary data are expected in a couple of months.

Key research achievements

1. AKR1B10 silencing reduces fatty acid and lipid synthesis in breast cancer cells
2. AKR1B10 silencing inhibits cell growth
3. AKR1B10 silencing suppresses tumorigenesis of breast cancer cells in female nude mice
4. AKR1B10 is overexpressed in primary breast cancer
5. AKR1B10 is overexpressed in recurrent breast cancer
6. AKR1B10 is overexpressed in DCIS
7. AKR1B10 is overexpressed in metastatic cancer cells in lymph nodes
8. AKR1B10 overexpression promote breast tumor growth
9. AKR1B10 overexpression promote lymph node metastasis of breast cancer
10. AKR1B10 overexpression resulting in worse patient survival.

Reportable outcomes

1. A manuscript enclosed in Appendix
2. A patent application for AKR1B10 as a biomarker of breast cancer (under preparation)

Conclusion

This study has demonstrated the overexpression of AKR1B10 in breast cancer, including DCIS, infiltrating carcinoma, recurrent tumors, and metastatic lymph nodes. AKR1B10 expression leads to tumor growth, lymph node metastasis, and worse prognosis, and thus AKR1B10 may be a novel specific marker and therapeutic target for breast cancer. These study results produced a manuscript submitted for publication, and a patent application for AKR1B10 as a marker for breast cancer.

Appendix: AKR1B10 manuscript

Overexpression of Aldo-Keto Reductase Family 1 B10 in Breast Cancer Is Associated with Tumor Growth, Lymph Node Metastasis, and Patient Survival

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Running title: AKR1B10 as a new risk factor for breast cancer

Abbreviations used: ACCA, acetyl-CoA carboxylase-α; AKR1B10, aldo-keto reductase family 1 member B10; DCIS, ductal carcinoma in situ; ER, estrogen receptor; HER-2, human epidermal growth factor receptor 2; and PR, progesterone receptor.

Key words: Aldo-keto reductase family 1 member B10, breast cancer, prognosis, tissue microarray, disease-related survival.

Abstract

Aldo-keto reductase family 1 member B10 (AKR1B10) is upregulated with tumorigenic transformation of human mammary epithelial cells. This study demonstrated that AKR1B10 was undetectable or at a very low level in normal breast lobules and ducts, but overexpressed in 20 (71.4%) of 28 ductal carcinomas in situ, 184 (83.6%) of 220 infiltrating carcinomas, and 28 (87.5%) of 32 recurrent tumors. A tissue microarray comprising 246 breast cancer cases with a mean follow-up time of 20 years showed that AKR1B10 expression in malignant tissues positively correlated with tumor size (p=0.0012) and lymph node metastasis (p=0.0123), but reversely correlated with disease-related patient survival (p=0.0120), particularly in breast cancer at early stages (tumor size < 2 cm^3 without metastasis) (p=0.0270). Univariate (p=0.0077) and multivariate (p=0.0192) analyses indicated that AKR1B10 was a significant prognostic factor, alone or in combination with tumor size and lymph node status, for breast cancer. Silencing of *AKR1B10* in BT-20 human breast cancer cells inhibited cell growth in culture and tumorigenesis in female nude mice. Taken together, our data suggest that AKR1B10 is overexpressed in breast cancer and may promote tumor growth and progression, thus being a novel prognostic marker and potential therapeutic target for this deadly disease.

Introduction

Breast cancer is the most common malignant disease and a leading cause of cancer deaths in women (Jemal et al 2009). Targeted therapies, such as endocrinal therapy for estrogen receptor (ER)- and/or progesterone receptor (PR)-positive tumors and immunotherapy for epidermal growth factor receptor 2 (HER2)-positive carcinomas, have significantly improved clinical outcomes of breast cancer (Dowsett et al 2006, Dowsett et al 2008, Sehdev et al 2009, Slamon et al 1987). However, effective therapeutic modalities are lacking for patients who are not suitable for or are resistant to these targeted therapies, such as triple negative breast cancer (Tan and Swain 2008). Another sophisticated issue in clinical management of breast cancer is the heterogeneity of disease and resultant difficulty for the design of a proper treatment in view of the host toxicity and costliness of current treatment modalities (Morris and Carey 2007). These clinical dilemmas call for new breast cancer-specific molecular markers for targeted therapy or for accurate prediction of outcomes and choice of treatment strategies.

Aldo-keto reductase family 1 member B10 (AKR1B10), also known as aldose reductase-like-1 (ARL-1), is a protein identified from human hepatocellular carcinomas (Cao et al 1998). As a reductase, AKR1B10 can efficiently detoxify dietary and cellular α, β-unsaturated carbonyls at physiological levels, protecting host cells from carbonyl toxicity (Yan et al 2007, Zhong et al 2009, Zu et al 2007). AKR1B10 is also active to *all-trans*-retinal, a precursor of the signaling molecule retinoic acid that regulates cell proliferation and differentiation, and to polycyclic aromatic hydrocarbon, an environmental pro-carcinogen (Crosas et al 2003, Gallego et al 2007, Quinn et al 2008). Recent studies from our laboratory have shown that AKR1B10 is upregulated with malignant transformation of human mammary epithelial cells (HMEC) and promotes cell growth and survival via mediating lipid metabolism (Ma et al 2008, Wang et al 2009). Therefore, AKR1B10 may play a critical role in cancer development and progression.

In humans, AKR1B10 is primarily expressed in the normal colon, small intestine and adrenal gland, but overexpressed in several tumors, including liver, lung, cervical and endometrial cancers (Cao et al 1998, Fukumoto et al 2005, Hyndman and Flynn 1998, Scuric et al 1998, Yoshitake et al 2007). In lung carcinoma, AKR1B10 is induced by cigarette smoke, participating in polycyclic aromatic hydrocarbon activation in smoke and malignant development of interstitial pneumonia in smokers (Fukumoto et al 2005, Kim et al 2007, Li et al 2008, Quinn et al 2008). In cervical cancer, AKR1B10 expression is associated with tumor recurrence after surgery and keratinization of cervical squamous cell carcinoma (Yoshitake et al 2007). In this study, we found that AKR1B10 is overexpressed in breast cancer and may promote tumor progression. In infiltrating breast carcinomas, AKR1B10 expression was correlated with tumor size, lymph node metastasis, and disease-related patient survival. In human breast cancer cells BT-20, AKR1B10 knockdown led to growth inhibition and suppression of tumorigenesis in female nude mice. This study unveiled that AKR1B10 is a novel prognostic factor and potential therapeutic target for human breast cancer.

Results

AKR1B10 is overexpressed in breast cancer.

Tissue microarray YTMA-23 consisted of 246 cancerous breast tissues from different patients. After immunohistochemistry, 220 malignant tissues had a quality of histology and immunohistochemistry and were evaluated for AKR1B10 expression. Figure 1A shows representatives of AKR1B10 expression in normal and primary malignant breast tissues. As summarized in Table 1, AKR1B10 was overexpressed in 184 (83.6%) of 220 breast cancer tissues, scored between '1' to '3' based upon staining strengths. A similar result was obtained from another tissue microarray (CC08-01-006) from a different source, in which 52 (85.2%) of 61 breast cancer tissues were stained positively, indicating a universal event of AKR1B10 overexpression in breast tumors.

To obtain more comprehensive understanding of AKR1B10 expression in breast cancer, we expanded this observation to other breast tumors. As shown in Figure 1B, AKR1B10 was overexpressed in DCIS (71.4%) and recurrent breast tumors (87.5%). AKR1B10 was also expressed in metastatic cancer cells in lymph nodes, with a high correlation with the primary breast tumors ($r=0.77$, $p<0.0001$). Table 1 summarizes the details of AKR1B10 expression in these tumors.

AKR1B10 expression positively correlates with tumor size and lymph node metastasis, but inversely with patient survival.

Cases in the tissue microarray YTMA-23 had complete clinical records and a mean follow-up time of 20 years and thus were analyzed for the correlation of AKR1B10 expression with clinicopathological parameters. As shown in Table 2, AKR1B10 expression in 220 interpretable breast tumors was positively correlated with the tumor size ($p=0.0012$) and lymph node metastasis ($p=0.0123$), but not with the patient age, tumor type, and nuclear grade. The tumors with AKR1B10 expression scored as '3' were approximately 1.3 times larger than those scored as '1' and 1.6 times than those as '0'. In 184 patients with an AKR1B10 positive tumor, 112 (60.9%) had regional node metastasis, compared to 8 (22.2%) of 36 AKR1B10-negative patients. In this data collection, AKR1B10 expression was not correlated with ER, PR or HER-2 alone or in any combinations.

The effect of AKR1B10 expression in cancer tissue on patient survival was evaluated with Kaplan-Meier plots, and results showed that AKR1B10 expression was negatively correlated with overall patient survival ($n=220$, $p=0.0026$) and in particular, the disease-related survival ($n=109$, $p=0.0120$) (Figures 2A and B). Univariate and multivariate analyses supported this result. In univariate analysis, tumor size (HR, 1.76; 95% CI, 1.28-1.43; $p=0.0006$), node status (HR, 1.77; 95% CI, 1.32-2.39; $p=0.0001$), or AKR1B10 expression (HR, 1.59; 95% CI, 1.13-2.24; $p=0.0077$) alone was significant predictors of patient survival, but tumor grade and ER, PR, or HER-2 status were not (Table 3). Multivariate analysis, in which tumor size, grade, node status, and AKR1B10, ER, PR and HER-2 expression were all taken into consideration, also supported AKR1B10 as a significant prognostic factor (HR, 1.2; 95% CI, 1.03-1.40; $p=0.0192$).

Breast cancer at early stages usually has better prognosis and therefore, prognostic markers are more critical for the design of a proper treatment strategy. In this data pool, 35 patients had a tumor at < 2 cm^3 without lymph node and distant metastasis and thus were designated at stage I (early stage) according to the TNM (primary tumor size, node metastasis and distant metastasis) classification. AKR1B10-related survival analyses indicated that in patients with an AKR1B10-negative tumor, the 25-year survival rate was higher than 90% compared to ~ 50% for patients with an AKR1B10-positive tumor ($p=0.0270$) (Figure 2C). Together these data suggest that AKR1B10 may be a valuable prognostic marker for breast cancer, particularly for those at early stages.

AKR1B10 silencing inhibited cell growth in couture and tumorigenesis in female nude mice.

Clinical data above indicate that AKR1B10 may promote the growth and progression of breast cancer. To further confirm this finding, we silenced *AKR1B10* in human BT-20 breast cancer cells. As shown in figure 3, *AKR1B10* silencing resulted in ~ 50% decrease of lipid synthesis, particularly in phospholipids critical for biomembrane synthesis, and remarkable inhibition of cell growth ($p<0.001$). The inhibition of lipid synthesis and cell growth induced by *AKR1B10* silencing in turn noticeably suppressed the tumorigenesis of BT-20 cells in female nude mice ($p=0.0462$) (Figure 4). The BT-20 cells with *AKR1B10* silencing did not form any tumors in 6 female nude mice until 42 days while the scrambled siRNA control cells inoculated in the same mice all produced tumors within 25 days (a ratio of 6/0 of scrambled siRNA cells/AKR1B10 siRNA cells). It is noteworthy that BT-20 cells are tumorigenic, but the tumor growth is relatively slow. Therefore, the tumor in Figure 4 was small.

Discussion

Breast cancer is a leading cause of cancer deaths in women. Due to the heterogeneity and high mortality of advanced breast cancer, the clinical outcomes remain poor; novel tumor-specific markers are needed for improving the clinical management of this deadly disease. This study revealed that AKR1B10 was overexpressed in breast cancer and may be a novel prognostic factor and therapeutic target for this disease.

AKR1B10 was overexpressed in >80% primary breast cancerous tissues from different sources, and clinicopathological and experimental data showed that AKR1B10 appeared to promote breast cancer growth and progression, resulting in worse patient survival. This could be explained by the known biological function of AKR1B10. Electrophilic carbonyls, which are constantly produced during cell metabolism, are highly cytotoxic, inducing protein dysfunction and DNA damage (Jacobs and Marnett 2010, LoPachin et al 2009). AKR1B10 can efficiently detoxify cellular α, β-unsaturated carbonyls and their glutathione conjugates at physiological levels (Zhong et al 2009). In addition, lipogenesis is critical to cancer cell growth and division; and increased lipogenesis and lipogenic enzyme expression, such as fatty acid synthase and ACCA, are early events occurring in cell carcinogenesis and cancer development (Rossi et al 2003, Witters et al 1994). AKR1B10 has been identified as a novel regulator of fatty acid and lipid synthesis via interacting with ACCA. In HCT-8 and NCI-H460 cells silencing of *AKR1B10* results in cell growth inhibition, susceptibility to carbonyls, and apoptotic death (Ma et al 2008, Wang et al 2009). In this study, we found that the silencing of *AKR1B10* in breast cancer cells BT-20 led to reduction of cellular lipid synthesis by ~ 50% and significant inhibition of cell growth. More importantly, *AKR1B10* silencing considerably suppressed the tumorigenesis (at 6/0 of scrambled siRNA cells/AKR1B10 siRNAcells) of the BT-20 cells in female nude mice, supporting the role of AKR1B10 in breast cancer development and progression.

During metastasis and recurrence, a cancer cell must evade host surveillance or adjuvant therapies and survive in a new microenvironment (Gupta and Massague 2006). During this process, AKR1B10 may confer a survival advantage to breast cancer cells by releasing carbonyl stress and promoting lipid metabolism, thus facilitating tumor metastasis and recurrence. Factually, AKR1B10 was overexpressed in lymph node metastatic and recurrent breast tumors, which supports this hypothesis.

In past decades, identification and validation of ER, PR and HER2 as molecular markers and therapeutic targets for breast cancer has greatly improved the patient survival. However, effective modalities for triple negative breast cancer or patients who are resistant to these targeted therapies are lacking (Slamon et al 1987, Tan and Swain 2008). Other breast cancer biomarkers currently identified, such as cancer antigen (CA) 15-3, carcinoembryonic antigen (CEA), ki-67, topoisomerase IIa, and oncotype DX, lack either disease-related specificity or validated clinical values (Conlin and Seidman 2007, Duffy 2006, Levenson 2007, Mariani et al 2009, Pritchard et al 2008, Urruticoechea et al 2005). This study unveiled AKR1B10 as a novel prognostic factor and potential therapeutic target, having significance in developing novel therapeutic strategies. This study also revealed that AKR1B10 was overexpressed in DCIS. Unfortunately, clinical data for DCIS patients were unavailable; it would be interesting to know whether AKR1B10 would affect the prognosis of these DCIS patients. Nevertheless, AKR1B10 expression in DCIS and infiltrating breast cancer at early stages may suggest its potential as a marker for breast cancer screening and early detection, warranting a further study.

In summary, this study reported for the first time that AKR1B10 is overexpressed in DCIS, infiltrating carcinoma, and lymph metastatic and recurrent tumors and acts as a negative prognostic factor. This finding may render AKR1B10 as a novel therapeutic target for breast cancer, and an extensive study is merited.

Materials and Methods:

Tissue microarrays and clinical data: In this study, 5 breast cancer tissue microarrays (TMA) were investigated for AKR1B10 expression: TMA-CC08-01-006 with 63 breast carcinomas (Biomax, MD), TMA-BR10010 containing 50 pairs of breast cancer and matching metastatic lymph nodes (Cybrdi, MD), YTMA (Yale Tissue Microarray)-89 consisting of 54 recurrent breast tumors, and YTMA-77 having 81 ductal carcinomas in situ (DCIS). In addition, the tissue microarray YTMA-23 contains 246 primary breast cancer tissues from patients diagnosed from 1962 to 1983, with complete clinicopathological records and a mean follow-up time of 20 years (Table S1); the expression state of ER, PR, and HER-2 in these tissues were determined (Giltnane et al 2009). All YTMAs were obtained from Yale TMA facility, Department of Pathology, Yale University School of Medicine, New Haven, CT. Normal breast tissues were included in all TMAs for an internal control.

Immunohistochemistry: AKR1B10 in normal and cancerous breast tissues was examined by immunohistochemistry. Briefly, after being dewaxed and hydrated, tissue microarray slides were immerged into preheated citric acid buffer (pH 6.5) at 90-95^{o}C for 20 min with microwaving. After being blocked with 5% horse serum for 30 min, slides were incubated with a specific rabbit anti-AKR1B10 antibody (1:2-5) (Ma et al 2008) at 4^{o}C in a humid box overnight. Thereafter, slides were washed 3 times and then incubated with HRP-conjugated secondary antibody (1:800; Pierce, IL) at room temperature for 1 hour. Enhanced DAB solution (Pierce, IL) was used to visualize signals. Staining intensity was evaluated blindly by a minimum of one researcher and one pathologist and scored from '0' to '3', representing no staining ('0'), low staining ('1'), intermediate staining ('2'), or high staining intensity ('3').

Cell culture and AKR1B10 silencing: Human breast cancer cells BT-20 were purchased from American Type Culture Collection (ATCC; Manassas, VA) and maintained in DMEM medium (Hyclone, UT) containing 10% fetal bovine serum (FBS) and 100U/ml penicillin at 37^{o}C, 5% CO_2. Scrambled or AKR1B10 siRNAs were chemically synthesized (Ambion, TX) and delivered into BT-20 cells; AKR1B10 knockdown was examined using Western blot, as previously described (Yan et al 2007).

Cell growth: Viable cells were measured using Alamar blue (ABD Serotec, UK) assays (Ahmed et al 1994). Briefly, cells (2000 cells/well) transfected with scrambled or *AKR1B10* siRNAs were plated in 96-well plates. Medium was added with 1/10 (v/v) Alamar blue and changed regularly every 24 hours. At days 1, 2, 3, 5, 6 and 7, reduced Alamar blue was detected at 590nm with a florescent spectrum (Thermo, CA). Viable cells correlate with the magnitude of Alamar blue reduction (%). Relative cell numbers were calculated following the manufacture's instructions.

Lipid synthesis: Cells in 12-well plates were pulsed with 1 μCi of [2-^{14}C]-acetate (53 mCi/mmol; Amersham Biosciences, CA) per well for 4 hours at 37°C, 5% CO2. Total lipids were extracted as described previously (Ma et al 2008). An aliquot (10 μl) of extracts was subjected to radioactivity measurements to determine the total lipids newly synthesized. Acetate incorporation into different lipid species, including free fatty acids, phospholipids, triglycerides, and cholesterol, was analyzed by TLC. Lipid extracts and appropriate lipid standards were spotted on silica gel (60 Å; Sigma, MO). After being air-dried, plates were developed in hexane/diethyl ether/acetic acid (70/30/1, v/v) to separate neutral lipids or in chloroform/methanol/acetic acid (65/25/10, v/v) to separate phospholipids. After being visualized by coloration in a staining solution (0.12 M NaCl, 20% methanol, and 300 mg/liter Coomassie Blue), lipids were collected and dissolved in 50% methanol. Radioactivity was measured by a scintillation counter (Beckman Instruments, CA). Values were normalized for protein contents (CPM/μg of protein).

Tumorigenesis in female nude mice: Scrambled siRNA or AKR1B10 siRNA1 was introduced into BT-20 cells (1×10^6) as described above. After incubation for 24 hours, cells were trypsinized and suspended in the mix of medium and equal volumes of Matrigel (BD Bioscience, CA) at 2×10^6/ 100 μl. Cells suspensions (50 μl/inoculation) were subcutaneously injected with a 25-gauge needle into mammary fat pads of female nude mice at 3 weeks old, one side for scrambled siRNA cells and the other for AKR1B10 siRNA1. One 17β-estradiol tablet (75 mg; Innovative Research, FL) was embedded subcutaneously in the neck of each mouse. Tumor formation was monitored every 2 days.

Statistical analysis: Descriptive statistics were examined for all variables. Non-parametric statistical tests were employed to examine the relationships between AKR1B10 expression and the other variables. Specifically, Spearman rank correlation coefficients were used to assess the relationship between AKR1B10 expression and continuous or ordinal variables; Wilcoxon rank-sum tests or Kruskal-Wallis tests were utilized for categorical variables. Kaplan-Meier survival curves were produced to examine the relationship between AKR1B10 expression and patient survival, as well as tumor formation in female nude mice, and log-rank test was used for statistically significant tests. A Cox proportional hazard regression model was employed in univariate and multivariate analysis. An unpaired *t* test was used for the tests of lipid synthesis and tumor volumes in animals, and cell growth was tested with two-way ANOVA. Results were considered statistically significant for $p<0.05$.

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Table 1. AKR1B10 expression in human breast tumors.

Breast cancer tissue microarrays from different sources were immunohistochemically stained and analyzed as described in the Materials and Methods. DCIS, ductal carcinoma in situ.

Tissue Microarrays	Description	Interpretable cases/ Total	AKR1B10 expression levels (%)			
			3	2	1	0
YTMA-23	Breast Cancer	220/246	33 (15.0)	69 (31.4)	82 (37.3)	36 (16.4)
YTMA-77	DCIS	28/81	4 (14.3)	6 (21.4)	10 (35.7)	8 (28.6)
YTMA-89	Recurrent Tumors	32/54	3 (9.4)	10 (31.3)	15 (49.6)	4 (12.5)
CC08-01-006	Breast Cancer	61/63	11 (18.0)	20 (32.8)	21 (34.4)	9 (14.8)
BR10010	BC/ w Met. LN [1]	50/50	8 (16.0)	14 (28.0)	21 (42.0)	7 (14.0)

[1] Breast cancer with self-paired metastasized lymph nodes

Table 2. Correlation of AKR1B10 expression with clinicopathological parameters.

Data were from the tissue array YTMA-23 in which 220 breast cancer cases were interpretable and had complete clinical records. ER, estrogen receptor; HER-2, human epidermal growth factor receptor 2; and PR, progesterone receptor.

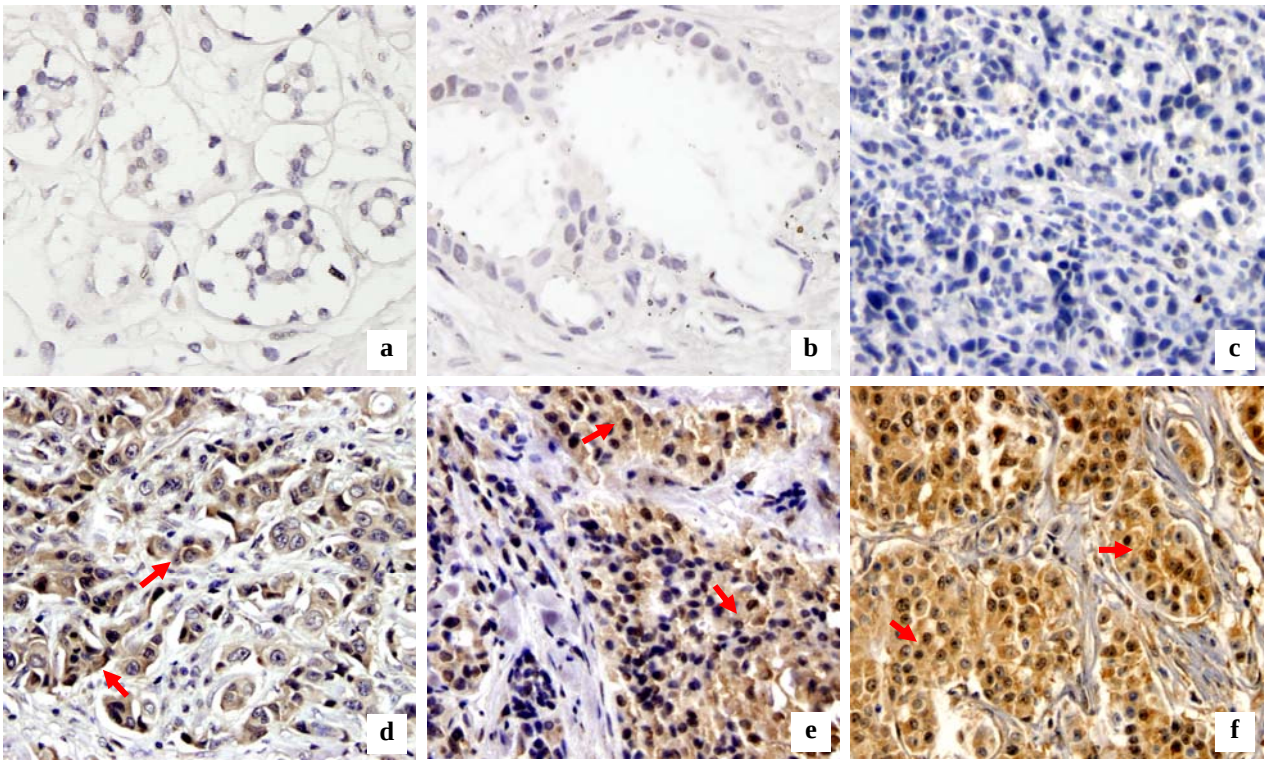
	AKR1B10 expression level (%)				p-value
Variables	3	2	1	0	
Subtotal	33 (15.0)	69 (37.3)	82 (37.3)	36 (16.4)	
Age (years)					
Mean (range)	58.3 (33-79)	57.8 (35-86)	61.3 (24-83)	60.7 (33-83)	0.5534
>50 (%)	22 (12.9)	57 (33.5)	69 (40.6)	22 (13.0)	
≤50 (%)	11 (22.0)	12 (24.0)	13 (26.0)	14 (28.0)	
Tumor Type					
Collid (%)	0	2 (22.2)	4 (44.4)	3 (33.3)	0.6730
Ductal (%)	19 (21.8)	27 (31.0)	34 (39.1)	5 (5.7)	
Lobular (%)	2 (6.1)	13 (39.4)	15 (45.5	3 (9.1)	
Tumor Size (cm^3)					
Mean(range)	2.8 (0.8-7.0)	2.8 (0.7-8.0)	3.7 (0.5-14.5)	2.5 (0.4-5.5)	0.0012
> 2 (%)	21 (15.3)	36 (26.3)	62 (45.3)	18 (13.1)	
≤ 2 (%)	12 (14.5)	33 (39.8)	20 (24.1)	18 (21.7)	
Node Metastasis					
Positive (%)	20 (16.9)	39 (33.1)	51 (43.2)	8 (6.8)	0.0123
Negative (%)	13 (12.9)	30 (29.7)	31 (30.7)	27 (26.7)	
Nuclear Grade					
1 (%)	3 (10.7)	8 (28.6)	8 (28.6)	9 (32.1)	0.0822
2 (%)	16 (13.9)	38 (33.1)	49 (42.6)	12 (10.4)	
3 (%)	14 (20.0)	22 (31.4)	24 (34.3)	10 (14.3)	
ER					
Positive (%)	18 (16.4)	35 (31.8)	40 (36.4)	17 (15.5)	0.8680
Negative (%)	15 (13.6)	34 (30.9)	42 (38.2)	19 (17.3)	
PR					
Positive (%)	13 (13.1)	31 (31.3)	37 (37.4)	18 (18.2)	0.4143
Negative (%)	20 (16.5)	38 (31.4)	45 (37.2)	18 (14.9)	
HER-2					
Positive (%)	20 (14.4)	44 (31.7)	50 (36.0)	25 (17.9)	0.6871
Negative (%)	13 (16.0)	25 (30.9)	32 (39.5)	11 (13.6)	

Table 3. Univariate and multivariate Cox Proportional analysis.

ER, estrogen receptor and HER-2, human epidermal growth factor receptor 2. P values refer to likelihood ratio test (LRT) for univariate analysis and Wald test for multivariate analysis.

	Univariate Analysis			Multivariate Analysis		
	Case #	Risk Ratio (95% CI)	p	Case #	HR (95% CI)	P
Tumor Grade (1/2 vs. 3)	215	1.13 (0.73-1.76)	0.5922	210	1.05 (0.82-1.35)	0.6944
Tumor Size (T1 vs. T2)	212	1.76 (1.28-2.43)	0.0006	210	1.13 (1.06-1.21)	0.0001
Node status (positive vs. negative)	238	1.77 (1.32-2.39)	0.0001	210	1.57 (1.13-2.18)	0.0070
ER (0/1 vs. 2/3)	239	0.94 (0.70-1.26)	0.6873	210	0.99 (0.87-1.13)	0.8775
HER-2 (0/1 vs. 2/3)	239	1.07 (0.71-1.62)	0.7473	210	0.96 (0.82-1.13)	0.6614
AKR1B10 (0/1 vs. 2/3)	218	1.59 (1.13-2.24)	0.0077	210	1.20 (1.03-1.40)	0.0192

A) Normal breast and primary breast cancer



B) DCIS, recurrent tumor, and lymph node metastasis

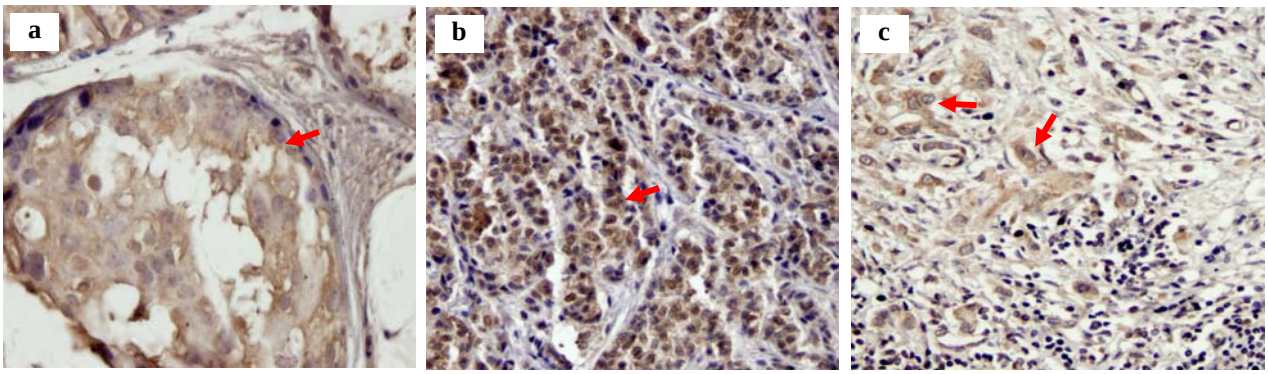
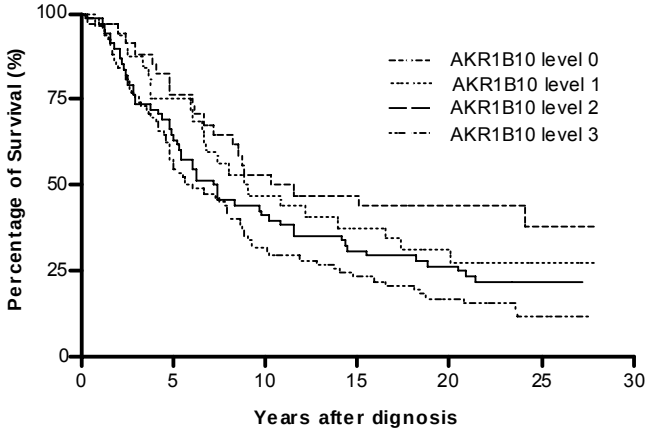
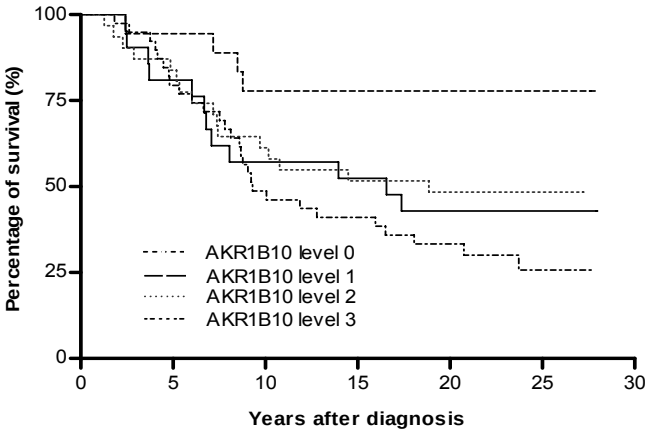


Figure 1. AKR1B10 expression in breast cancer tissues. AKR1B10 expression in normal and malignant breast tissues was examined by immunohistochemistry as described in the Materials and Methods. Results were reviewed blindly by a researcher and a pathologist. (A) AKR1B10 expression in normal breast lobules (a) and ducts (b) and in breast cancers, scored at '0' (c), '1' (d), '2' (e), and '3' (f), respectively. (B) AKR1B10 expression in ductal carcinoma in situ (DCIS) (a), recurrent tumor (b) and metastatic breast cancer cells in lymph node (c)**.** Arrows denote AKR1B10 expression cancer cells.

A) Overall survival



B) Disease-related survival



C) TNM Stage 1 tumor survival

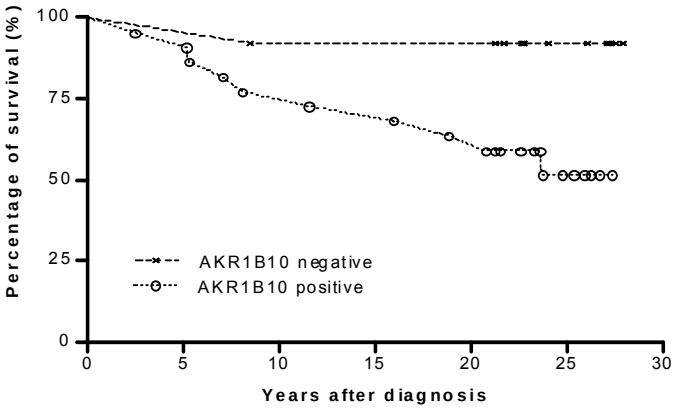


Figure 2. Kaplan-Meier analysis of patient survival. Data were from YTMA-23 consisting of 220 interpretable breast cancer cases with a mean follow-up time of 20 years. (A) Overall survival (n=220, p=0.0026), in which all breast cancer patients who died or survive were included. (B) Disease-related survival (n=109, p=0.0120), in which only patients who died from breast cancer or are disease-free survivals were counted. (C) Disease-related survival of patients with a breast cancer at early stages (n=35, p=0.0270), in which only patients at TNM stage 1 (tumor size < 2cm and lymph node negative) were grouped.

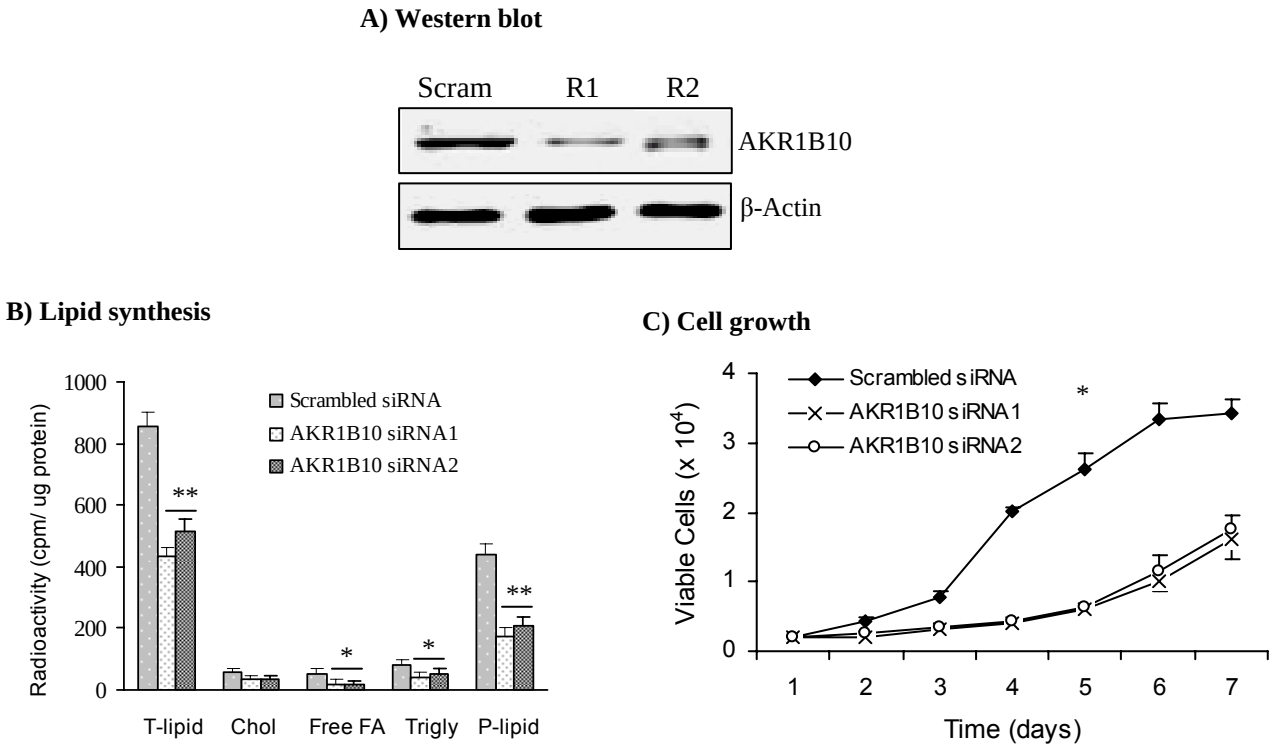
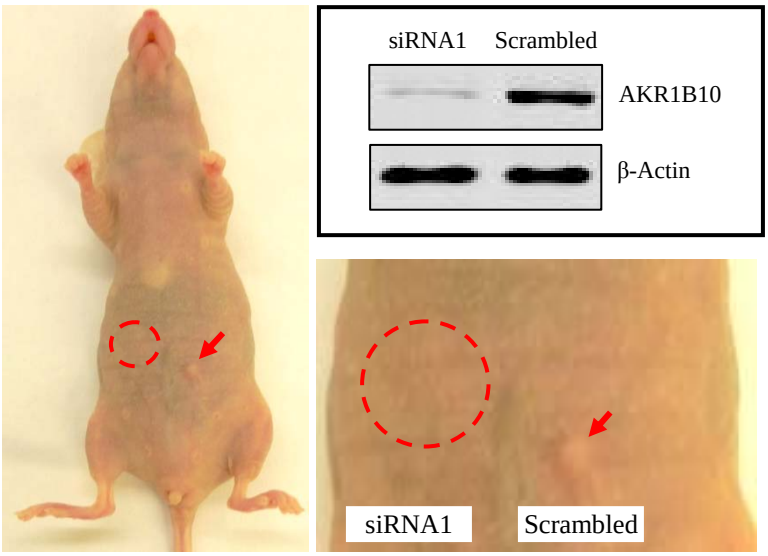


Figure 3. Inhibition of lipid synthesis and cell growth by *AKR1B10* silencing. Scrambled or AKR1B10 siRNA1/2 were introduced into human BT-20 breast cancer cells as described in Materials and Methods. (A) Western blot, showing specific AKR1B10 knockdown by two siRNAs targeting the encoding or non-encoding regions of AKR1B10. R1, AKR1B10 siRNA1; R2, AKR1B10 siRNA2. (B) Lipid synthesis, detected by [2-^{14}C]-acetate incorporation. * $p<0.05$ and ** $p<0.001$ compared to scrambled siRNA control cells. T-lipid, total lipid; Chol, cholesterol; FA, fatty acids; Trigly, triglyceride; and p-lipid, phospholipid. (C) Cell growth, detected by Alamar blue. * $p<0.001$ compared to the scrambled siRNA control cells.

A) Tumor images



B) Kaplan-Meier plots

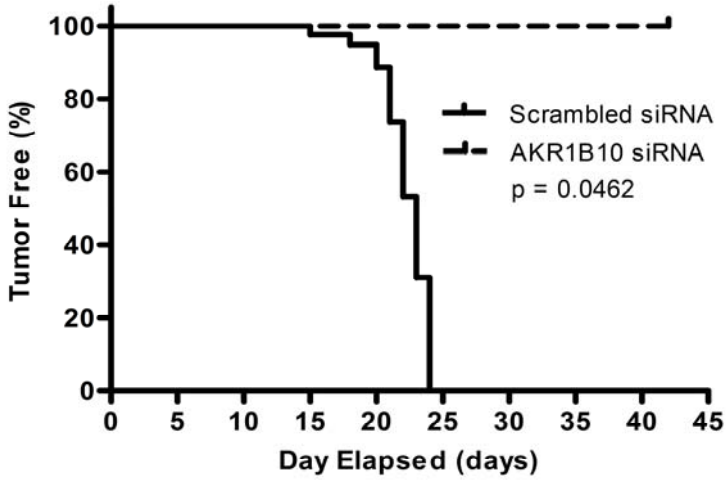


Figure 4. Suppression of tumorigenesis of BT-20 cells by *AKR1B10* silencing. *AKR1B10* silencing in BT-20 cells, cell inoculation, and tumor monitoring were conducted as described in Materials and Methods. (A) Tumor formation in female nude mice. In frame: Western blot of AKR1B10 knockdown in BT-20 cells. The arrow denotes the tumor formed from scrambled siRNA cells, and the circle indicates the area where AKR1B10-silencing cells were inoculated. (B) Kaplan-Meier plots. n=6 inoculations each for scrambled or AKR1B10 siRNA cells; p=0.0462. Scrambled, scrambled siRNA; siRNA1, AKR1B10 siRNA1.